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Original Paper

Renal Mesangial Cells Isolated from Sphingosine Kinase 2 Transgenic Mice Show Reduced Proliferation and are More Sensitive to Stress-Induced Apoptosis

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Key Words
Mesangial cells • Proliferation • Apoptosis • Autophagy • S1P • Transgenic SK-2 mice

Abstract
Background/Aims: Sphingosine 1-phosphate (S1P) is considered as a key molecule regulating various cell functions including cell growth and death. It is produced by two sphingosine kinases (SK) denoted as SK-1 and SK-2. Whereas SK-1 has been extensively studied and has been appointed a role in promoting cell growth, the function of SK-2 is controversial, and both pro-proliferative and pro-apoptotic functions have been suggested. In this study we investigated whether renal mesangial cells isolated from transgenic mice overexpressing the human *Sphk2* gene (hSK2-tg) showed an altered cell response towards growth-inducing and apoptotic stimuli. ***Methods:*** hSK2-tg mice were generated by using a Quick Knockin^R strategy. Renal mesangial cells were isolated by a differential sieving method and further cultivated *in vitro*. Lipids were quantified by mass spectrometry. Protein expression was determined by Western blot analysis, cell proliferation was determined by ^{3}H-thymidine incorporation, and apoptosis was determined by a DNA fragmentation ELISA. ***Results:*** We show here that kidneys and mesangial cells from hSK2-tg mice express the hSK2 as well as the endogenous mouse mSK2. hSK2 and mSK2 predominantly resided in the cytosol of quiescent transgenic cells. However, S1P accumulated strongly in the nucleus and only minimally in the cytosol of transgenic cells. Functionally, hSK2-tg cells proliferated less than control cells under normal growth conditions and were also more sensitive towards stress-induced apoptosis. On the molecular level, this was reflected by reduced ERK and Akt/PKB activation, and upon staurosporine treatment, by a sensitized mitochondrial pathway as manifested by reduced anti-apoptotic Bcl-XL expression and increased cleavage of caspase-9, downstream caspase-3 and PARP-1. ***Conclusion:*** Altogether, these data demonstrate that SK-2 exerts an antiproliferative and apoptosis-sensitizing effect in renal mesangial cells which suggests that selective inhibitors of SK-2 may promote proliferation and reduce apoptosis and this may have impact on the outcome of proliferation-associated diseases such as mesangioproliferative glomerulonephritis.

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Introduction

Sphingosine kinases (SK) catalyze the formation of sphingosine 1-phosphate (S1P) which has turned out as a key regulator of a variety of cell responses such as cell growth and survivial, cell differentiation, migration, and inflammatory reactions [1-4]. SKs are ubiquitously expressed and exist as two subtypes, SK-1 and SK-2, and each of at least two splice variants [1]. The physiological functions of all these forms are presently not clear. At least, accumulating reports now suggest a key function for SK-1 in cell proliferation and migration [5]. Thus, an upregulation of SK-1 mRNA and protein has been shown in several histotypes of tumors and various tumor-derived cell lines [6-9]. In line with this, recently developed inhibitors of SK-1 have proven to be effective in reducing tumor cell growth *in vitro* and tumor growth in mice [6, 10] although the specificity of the inhibitors for SK-1 compared to SK-2 is still not proven. The function of SK-2 is to date still controversial as either pro-apoptotic function or anti-apoptotic and pro-proliferative functions have been forwarded. SK-2 contains a BH3 domain which was shown to interact with Bcl-X_L and thereby inhibit the anti-apoptotic potential of Bcl-X_L consequently results in cell apoptosis [11]. Furthermore, the overexpression of SK-2 in various murine and human cell lines blocks DNA synthesis [12, 13] and this in turn was abrogated by mutation of the nuclear localization signal (NLS) of SK-2 [12]. These latter studies suggested that especially nuclear SK-2 acts anti-proliferative whereas growth factors, through phosphorylation of SK-2, allow translocation of the enzyme to the cytosol and ablate its antiproliferative action, allowing them to trigger cell cycle progression via other mechanisms. Furthermore, serum deprivation of human colon cancer cells resulted in an upregulation of SK-2 mRNA expression [14]. In contrast, it was reported that in breast cancer cells, extracellular signal-regulated kinase (ERK) can phosphorylate SK-2 [15] and thereby increases its activity and cell migration [16]. In line with this, a putative specific SK-2 inhibitor ABC294640 showed anti-tumor activity *in vivo* in a xenograft mouse model [17]. Due to these contradictory reports about the role of SK-2 in cell growth and death, further studies are needed to clarify the function of SK-2 which may also be different depending on the cell type.

In this study, we have used mesangial cells isolated from mice that constitutively overexpresses the human SK-2 enzyme, and investigated the role of hSK2 overexpression on growth and death of mesangial cells. That transgenic cells showed reduced growth coupled to reduced ERK and Akt activation, and increased stress-induced apoptosis coupled to reduced expression of the anti-apoptotic Bcl-XL, and increased cleavage of the initiator caspase-9, the executer caspase-3 and PARP-1. These data strengthen the hypothesis of SK-2 as an antiproliferative and pro-apoptotic enzyme in renal mesangial cells, and suggest that selective inhibitors of SK-2 may have an impact on the outcome of proliferation-associated diseases such as mesangioproliferative glomerulonephritis.

Materials and Methods

Chemicals

[6-^{3}H]methyl-thymidine (specific activity: 20 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, US); γ-[^{32}P]-ATP (10 µCi/ 20 mM) was from PerkinElmer (Rodgau, Germany); the secondary antibodies, hyperfilm MP, and the enhanced chemiluminescence (ECL) reagents were from GE Health Care Systems GmbH (Freiburg, Germany); staurosporine, LPS, cycloheximide and the β-actin (clone AC-15) antibody were from Sigma Aldrich (Deisenhofen, Germany); TNFα was from PeproTech Inc. (Rocky Hill, NJ, US); antibodies against phospho-ERK1/2, phospho-Ser^{473}-PKB/Akt (9271), total Akt/PKB (9272), PARP-1 (9542), caspase-3 (9662), Bcl-XL (2762), calreticulin (2891) and cleaved caspase-9 (9509) were from Cell Signaling (Frankfurt, Germany); lamin B (C-20, sc-6216) and GAPDH (sc-20357) antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany); total ERK1 and ERK2 antibodies were generated as previously described [18]; polyclonal antibodies against mSK2 and hSK2 were generated as previously described [19]; the DNA fragmentation PLUS ELISA was from Roche Diagnostics (Mannheim, Germany);

the Nuclear Extraction Kit was from Abcam (Cambridge, U.K.); all cell culture nutrients were from Life Technologies (Karlsruhe, Germany).

Generation of a hSK2-Knockin mouse strain

The generation of this transgenic mouse strain was carried out by using the "Quick Knock-in™" targeting vector of Genoway (Lyon, France) as previously described [19]. These mice carry a LoxP-STOP-LoxP cassette in front of the transgene, which prevents transgene transcription (hSK2-ctrl). By cross-breeding these mice with ubiquitous CRE recombinase expressing mice (Ella-CRE), the floxed STOP cassette is excised to allow hSK2 transgene expression (further denoted hSK2-tg). Breeding and generation of transgenic mice were approved by the local Ethics Committee for Animal Research and adhered to standard guidelines.

Cell culturing

Renal mesangial cells were isolated from mice, characterized and cultivated as described [20]. Outgrown mesangial cells were subcultured and further used up to passage 16. Prior to stimulation, cells were incubated for 24 h with Dulbecco's modified Eagle medium (DMEM) containing 1% FBS. hSK2 overexpression was verified by Western blot analysis using a species-specific antibody against hSK2 which has no cross-reactivity with the endogenous mouse enzyme.

Cell stimulation and Western blot analysis

Stimulated cells were scraped into ice-cold lysis buffer and homogenized exactly as previously described [19]. Equal amounts of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane and subjected to Western blot analysis according to standard procedures.

[^{3}H]thymidine incorporation

2x 10^4 cells were plated per well of a 24-well plate and incubated in growth medium containing 0.2 µCi/ml of [^{3}H]methyl-thymidine. After different time periods, the medium was removed and cells were washed twice with PBS, and incubated with ice-cold 5% (w/v) trichloroacetic acid (TCA) for 30 min. Cells were washed twice with 5 % TCA and the DNA was solubilized in 1 M NaOH for 30 min at 37°C, and the radioactivity was counted in a β-counter.

DNA fragmentation assay

Cells in 96-well plates were stimulated as indicated and directly taken for detection of DNA fragmentation using a Cell Death detection ELISA^{PLUS} according to the manufacturer's instructions.

Nuclear extraction assay

Cells from two confluent 100-mm cell dishes were taken for a fractionation step into a nuclear and a cytosolic fraction using a nuclear extraction kit. Fractions were separated by SDS-PAGE, transferred to nitrocellulose membranes and subjected to Western blot analysis using antibodies as indicated in the figure legends.

SK-2 activity assay

In vitro SK-2 activity was assayed exactly as described [7, 21] by using 10 µg of protein lysates. Lipids were separated by thin layer chromatography and radioactivity incorporated into S1P was analysed by an Imaging system (Fuji, Düsseldorf, Germany).

Sphingolipid quantification by LC-MS/MS

Fractionated cytosolic (Cyt) and nuclear (Nuc) samples were scraped into methanol containing internal C17-ceramide, C17-sphingosine and C17-S1P standards and subjected to lipid extraction and LC/MS/MS analysis as previously described [22].

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

Results

In our previous studies using renal mesangial cells isolated from SK-2 gene deficient mice, we observed an increased proliferative phenotype and a protection from stress-induced apoptosis when compared to wildtype cells [23]. Therefore, in this study, we aimed in investigating whether a systemic overexpression of SK-2 in mice would lead to an opposite cellular phenotype, i.e. increased apoptosis and/or less proliferation.

By using the "Quick Knock-in" strategy of Genoway (Lyon, France), a transgene mouse strain was generated that overexpresses the human SK-2 (hSK2) gene under the control of a CAG promoter followed by a *LoxP*-STOP-*LoxP* cassette. This transgene mouse strain, denoted hSK2-ctrl does not overexpress hSK2 due to the incorporated STOP cassette. However, when mice were cross-bred with Cre-expressing mice, this STOP cassette is excised allowing activation of the hSK2 transgene expression (furtheron hSK2-tg).

To characterize the overexpression of hSK2 and compare it to the endogenous mSK2, we used two species-specific antibodies, one against the C-terminal sequence of mouse SK-2 and one against the N-terminal sequence of human SK-2 [19]. The specificities of these antibodies were confirmed in protein lysates of hSK-2 overexpressed HEK293 cells, human mesangial cells, rat mesangial cells and mouse mesangial cells either isolated from wildtype C57BL6 mice, SK-1 knockout or SK-2 knockout mice. As shown in Fig. 1, the hSK-2 antibody only detected the human enzyme at 66 kDa when overexpressed in HEK293 cells. No band was seen in lysates of human mesangial cells suggesting very low protein expression levels. Neither rat nor mouse SK-2 were detected with the hSK2 antibody. On the opposite, the mSK2 antibody did not detect the human enzyme but did detect rat and mouse SK-2 (Fig. 1). Therefore, these two antibodies can be used to discriminate between the transgenic hSK2 and the endogenous mSK2 expressions in Western blot analyses.

Kidneys and primary cultures of renal mesangial cells, isolated from wildtype or control transgenic mice (hSK2-ctrl) or mice with an active hSK2 transgene (hSK2-tg), were characterized by Western blot analysis showing that only in the active hSK2 transgenic mice, hSK2 is detected in both kidneys (Fig. 2A) and isolated renal mesangial cells (Fig. 2B). mSK2 was detected in all mice but was slightly reduced when hSK2 was overexpressed (Fig. 2A and B), suggesting some compensatory effect.

To investigate the subcellular localization of SK-2 in mouse mesangial cells, the nuclear fraction was separated from the remaining cytosol/particulate fraction (referred to as cytosol) by using a nuclear extraction kit. Notably, the remaining cytosol/particulate fraction also includes subcellular organelles such as the ER as shown by positive staining for the ER marker calreticulin (Fig. 3A). Western blot analysis of these fractions revealed that in the hSK2-tg cells, hSK2 is predominantly localized in the cytosol with only a small amount in the nucleus (Fig. 3A). A similar distribution

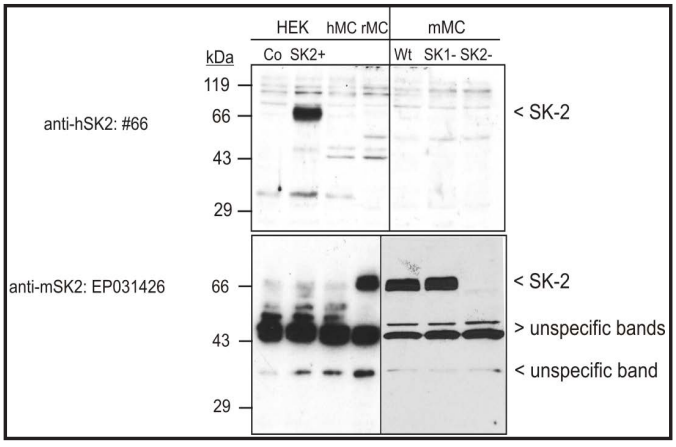

Fig. 1. Characterization of the species specificities of mSK-2 and hSK-2 antibodies in mesangial cells. Untransfected HEK293 cells (Co), or HEK293 cells overexpressing hSK-2 (SK2+), human (hMC), rat (rMC), and mouse mesangial (mMC) cells isolated from C57BL/6 mice (Wt), SK-1 deficient mice (SK1-) and SK-2 deficient mice (SK2-) were taken for protein extraction and separation, and subjected to Western blot analysis using antibodies against mSK-2 (EP031426; dilution 1:1000) (lower panel) or hSK-2 (#66; dilution 1:1000, upper panel). Bands were visualized by the ECL method according to the manufacturer's instruction.

was found for the endogenous mSK2 (Fig. 3A). Nuclear and cytosolic fractions were also taken for a SK-2 activity assay to see whether total SK-2 enzyme activity was increased in hSK2-tg cells. Indeed, as shown in Fig. 3B, the cytosolic fraction of hSK2-tg cells showed a several fold increase of SK-2 activity compared to hSK2-ctrl cells, whereas nuclear SK-2 activity did not change (Fig. 3B). To see whether transgenic cells have altered sphingolipid levels, S1P, sphingosine and C16-ceramide were quantified by mass spectrometry. hSK2-tg cells had significantly increased S1P in the cytosol, and even more pronounced in the nucleus when compared to the control cells (Fig. 3C). Sphingosine was also markedly increased in the nuclear fraction and moderately in the cytosol of transgenic cells whereas C16-ceramide was not altered (Fig. 3C).

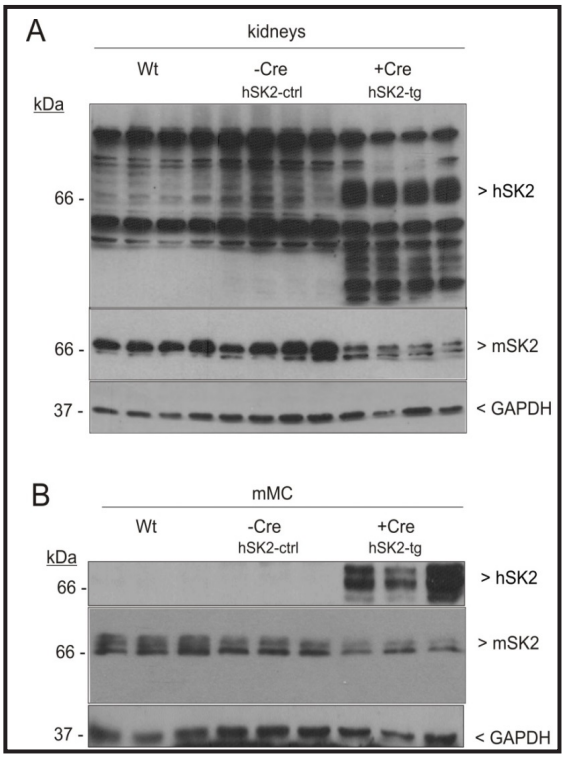

Fig. 2. Characterization of kidneys and isolated mesangial cells of hSK2-tg mice for transgene and endogenous SK-2 protein expression. Protein lysates of kidneys (A) and isolated mouse mesangial cells (B) from wildtype mice (Wt), hSK2–ctrl mice (expressing no Cre recombinase, -Cre), and hSK2-tg mice (hSK2tg, expressing Cre recombinase, +Cre), were separated by SDS-PAGE and subjected to Western blot analysis using antibodies against human SK-2 (hSK2, upper panels), mSK-2 (middle panels) and GAPDH (lower panels). Data show a representative blot from four (A) and three (B) mice for each group.

In a further attempt to study the effect of hSK2 overexpression on mesangial cells behaviour, incorporation of [^{3}H]thymidine into DNA was analysed. As shown in Fig. 4A, hSK2-tg cells proliferated significantly less than control cells and wildtype cells. hSK2-tg cells also showed reduced levels of phosphorylated ERK1/2 (Fig. 4B) and Akt/PKB (Fig. 4C) which are two signalling pathways tightly coupled to proliferation. Next, apoptosis of cells was measured by a DNA fragmentation ELISA. Apoptosis was induced by various stimuli such as staurosporine, a combined treatment of TNFα plus cycloheximide (TNFα/CHX) or LPS/CHX. Remarkably, hSK2 transgenic cells were more sensitive to all the tested stimuli (Fig. 5A and B) but it was most pronounced for staurosporine. On the molecular level, the increased apoptosis seen in hSK2-tg cells upon staurosporine treatment was detected by an increased PARP-1 cleavage (Fig. 5C), and by increased caspase-3 cleavage (Fig. 5D).

As apoptosis can occur through two main pathways, either through an extrinsic receptor-mediated one, or an intrinsic pathway involving mitochondria, which both culminate in the activation of the main executioner caspase-3, we further investigated whether the mitochondrial pathway including apoptosome protein complex formation and caspase-9 activation is involved. Notably, SK-2 was previously reported to act pro-apoptotic due to interaction with the survival factor Bcl-XL [11] and our previous data on SK-2 deficient mesangial cells had revealed an upregulation of Bcl-XL which coincides with reduced stress-induced apoptosis [24]. We therefore tested here whether Bcl-XL was altered in hSK2-tg cells. As seen in Fig. 5E, basal Bcl-XL protein expression was not changed by the sole overexpression of hSK2. Bcl-XL appears as a double band because of deamidation of two amino acids Asn^{52} and Asn^{65} into Asp. Thus, the most upper band represents the double deamidated form, which is the only form that is functionally characterized [25]. In mesangial

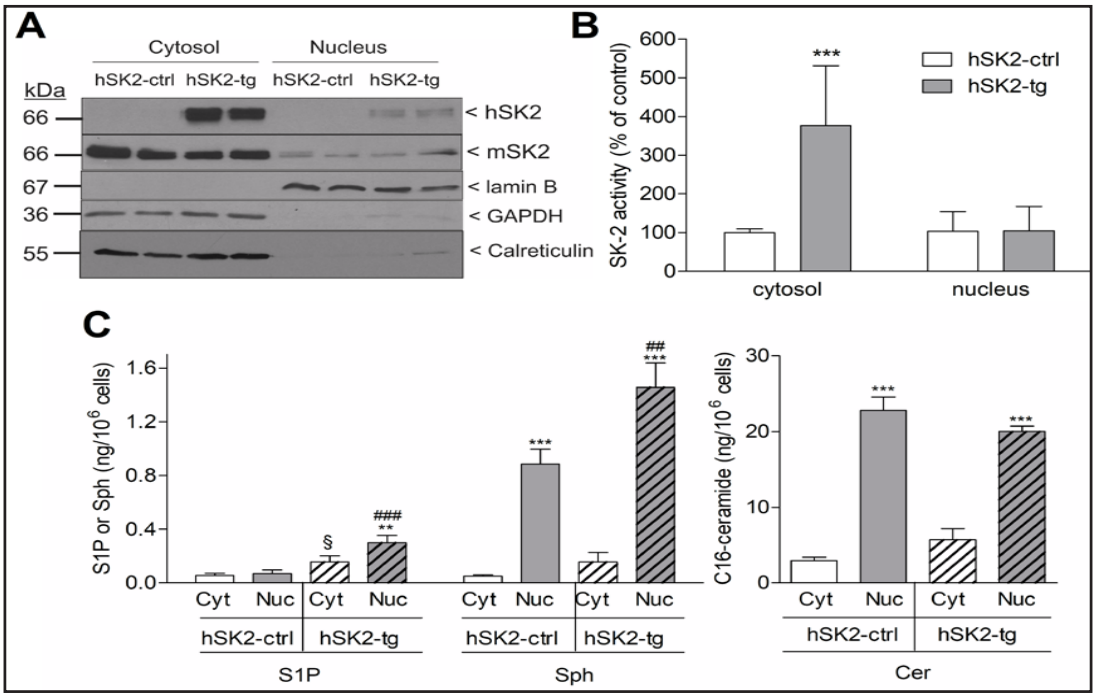

Fig. 3. Subcellular localization of hSK2 and mSK2, SK-2 activity and sphingolipid levels in transgenic mouse mesangial cells. (A) Nuclear and cytosolic fractions were prepared from control cells (hSK2-ctrl) and hSK2-tg cells as described in the Methods Section. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis using antibodies against hSK2 (for transgene detection), mSK2 (for endogenous enzyme detection), mSK-1, lamin B (for nuclear fraction detection), GAPDH (for cytosolic fraction detection) and calreticulin (for ER detection). Blot shows a representative experiment in duplicates. (B) Nuclear and cytosolic fractions were prepared from hSK-2-ctrl cells and hSK2-tg cells and equal amounts of protein were taken for a SK-2 activity assay as described in the Methods Section. Data are expressed as % of control cytosolic activity and are means ± S.D. (n=4), ***p<0.001 considered statistically significant when compared to hSK2-ctrl cytosolic values. (C) Cytosolic (Cyt) and nuclear (Nuc) fractions were taken for lipid extraction and mass spectrometric analysis of S1P, sphingosine (Sph) and C16-ceramide (Cer) as described in the Methods Section. Data are expressed as ng per 10^6 cells, and are means ± S.D. (n=3), **p<0.01, ***p<0.001 considered statistically significant when compared to the hSK2-tg cytosolic samples, §p<0.05 compared to hSK2-ctrl cytosolic samples; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared to hSK2-ctrl nuclear samples.

cells treated with staurosporine, all Bcl-XL protein is rendered deamidated and in addition, total protein is reduced consistent with previous studies in other cell types that staurosporine downregulates the antiapoptotic factor Bcl-XL [26]. In hSK2-tg staurosporine-treated cells, the total Bcl-XL is further downregulated (Fig. 5E).

Downstream factors coupled to Bcl-XL are Apaf-1 and the initiator caspase-9 [27]. In hSK2-tg cells, there was an increased basal cleavage of caspase-9 when compared to control cells, but staurosporine strongly induced the cleavage of caspase-9 which again, was several fold increased in hSK2-tg cells than in control cells (Fig. 5E).

Discussion

In this study we have used a transgenic mouse strain, which systemically overexpresses hSK2, in order to clarify the function of SK-2 which is so far rather controversial. We show here that renal mesangial cells isolated from these hSK2-tg mice have an anti-proliferative

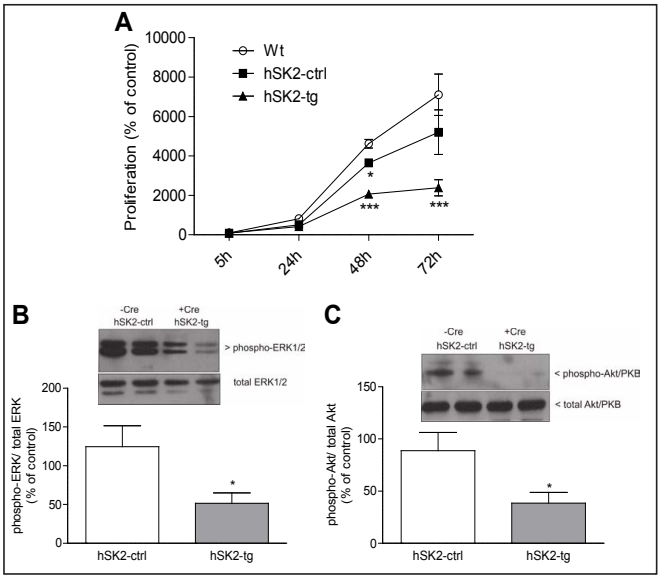

Fig. 4. Effect of hSK2 overexpression on cell proliferation (A), ERK (B) and Akt (C) phosphorylation in mouse mesangial cells. (A)2x 10^4 mouse mesangial cells from either wildtype C57BL/6 mice (Wt), hSK2-ctrl mice or hSK2-tg mice were seeded and grown for the indicated time periods in growth medium supplemented with [^{3}H]thymidine and thereafter processed as described in the Methods Section. Data are expressed as % of controls and are means ± S.D. (n=3). *p<0.05, ***p<0.001 considered statistically significant when compared to the respective Wt values. (B and C) Confluent and quiescent hSK2-ctrl cells and hSK2-tg cells were taken for protein extraction and separation, and subjected to Western blot analysis using antibodies against phospho-ERK1/2 (B, inset, upper panel) and total ERK1/2 (B, inset, lower panel), and phospho-Ser^{473}-Akt/PKB (C, inset, upper panel) and total Akt/PKB (C, inset, lower panel). Bands were visualized by the ECL method and densitometrically evaluated. Data are expressed as % of controls and are means ± S.D. (n=4), *p<0.05 considered statistically significant when compared to the hSK2-ctrl values.

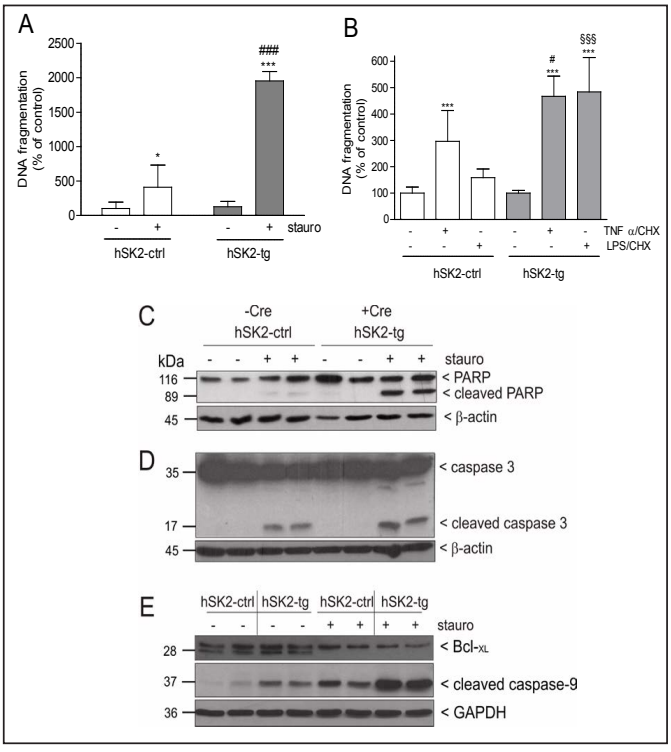

Fig. 5. Effect of hSK2 overexpression on DNA fragmentation, PARP cleavage, caspase-3 cleavage, Bcl-XL expression and caspase-9 cleavage in mouse mesangial cells (A)Quiescent mouse mesangial cells isolated from hSK2-ctrl mice or hSK2-tg mice were treated for 6 h with either vehicle (-) or 10nM staurosporine (stauro, +). (B) Cells were treated for 6 h with either vehicle (-), cycloheximide (CHX, 10μM) plus TNFα (2nM), or CHX plus LPS (100ng/ml). Thereafter, cells were taken for a DNA fragmentation ELISA as described in the Methods Section. Data are expressed as % of control and are means ± S.D. (n=4 (A) or 6 (B)), *p<0.05, ***p<0.001 considered statistically significant when compared to the respective vehicle-treated controls, #p<0.05, ###p<0.001 compared to hSK2-ctrl stauro (A) or hSK2-ctrl TNF/CHX (B) samples, §§§p<0.001 compared to hSK2-ctrl LPS/CHX samples.(C-F) Quiescent hSK2-ctrl cells and hSK2-tg cells were incubated for 6 h in either vehicle (-) or 10nM staurostorine (+). Thereafter, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis using antibodies against PARP (C), caspase-3 (D), Bcl-XL (E), and cleaved caspase-9 (E). β-actin (all lower panels) was used to verify equal loading. Bands were visualized by the ECL method. Results in C-E show representative blots of three independent experiments performed in duplicates.

phenotype. They grow slower in culture and are more sensitive to stress-induced apoptosis. Previously, we showed that a genetic knockout of SK-2 in mice led to an increased proliferative phenotype of mesangial cells in culture [23] and to a protection from stress-induced apoptosis of mesangial cells [24].

These data are in agreement with the previous report of Spiegel and coworkers [11] which showed that SK-2 possesses a BH3 domain which interacts with the Bcl-XL survival factor and, by this physical interaction, traps Bcl-XL and allows execution of apoptosis. By mutation of the BH3 domain, the pro-apoptotic effect of SK-2 was abolished [11]. In addition, SK-2 knockout mice showed an enhanced development of colitis-associated tumors which was explained by a compensatory upregulation of SK-1 and consequently, increased serum and colon S1P levels [28]. Our data are also in agreement with Igarashi et al [12]. and Okada et al [13]. who showed an anti-proliferative action of overexpressed SK-2 in cells.

In contrast, others have rather proposed an opposite function for SK-2, i.e. in promoting proliferation. Most of these studies of SK-2 however relied on one single putative SK-2 inhibitor, ABC294640, which was forwarded as being selective and specific [17]. Notably, the *in vitro* characterization of the inhibitor revealed a rather high IC_{50} value of 60 μM [17]. This inhibitor was reported to reduce plasma S1P levels and even more importantly, tumor tissue S1P levels in xenograft models [29]. Using this inhibitor in various tumor xenograft models revealed a great tumor reducing effect [17, 29-31]. However, as always with such compounds, unspecific effects on other targets, which could contribute to the observed therapeutic effects in those cancer models, must be considered. In this view, it was shown that ABC294640 can directly block the estrogen receptor thus giving a possible explanation why estrogen-dependent breast cancer cells, such as MCF7, were especially sensitive to the inhibitor. In addition, our previous data in mouse mesangial cells revealed that ABC294640 reduced DNA synthesis equally well in wildtype cells and in SK-2 knockout cells thus clearly demonstrating an unspecific effect independent of SK-2 [23]. Despite these emerging off-target effects of ABC294640, the compound was taken into a combined clinical phase I and IIa trial in patients with refractory/relapsed diffuse large B cell lymphoma (DLBCL).

Very recently, more potent SK-2 inhibitors were synthesized such as K145 [32], and pyrrolidyl guanidine compounds SLC5111312 and SLM6081442 [33]. Interestingly, application of these latter pyrrolidyl guanidine compounds to mice caused an increase of plasma S1P levels and this fits well to the situation of SK-2 knockout mice which also show enhanced plasma S1P levels [34]. The increased plasma S1P was explained with a dysregulated removal of plasma S1P that is mediated by SK-2 although mechanistically still unclear [33]. These novel series of SK-2 inhibitors will certainly need to be tested in tumor models to support the results obtained with the first generation compound ABC294640.

In an attempt to explain the controversial results of SK-2 on proliferation and apoptosis of cancer cells, Neubauer et al. proposed that the amount of SK-2 expression determines the cellular outcome [35]. This was based on studies performed in an inducible overexpression system where high-level overexpression of SK-2 in HEK293 cells (> 200-fold) reduced cell proliferation and survival, whereas low-level overexpression of SK-2 (in the range of 2-8-fold) promoted cell survival and proliferation [35]. Differences between the low- and high-SK-2 cells were found in the levels of ceramide subspecies and the subcellular localization of SK-2. Only high-SK-2 cells showed an increase of certain long-chain ceramide subspecies which may contribute to the apoptotic phenotype. Additionally, high-SK-2 cells express the enzyme mainly in the nucleus whereas low-SK-2 cells express the enzyme mainly in the cytosol and at the plasma membrane. The authors also showed that a modest (2.5-fold) upregulation of SK-2 occurs in a broad range of cancers which led them to suggest that indeed, inhibition of SK-2 may have an anti-cancer potential [35].

Interestingly, in our study we also detected a predominant expression of hSK2 protein in the cytosol/particulate fraction of transgenic cells (Fig. 3A), which coincided with an increased SK-2 activity (Fig. 3B). However, on the level of lipids, S1P, although being significantly increased in the cytosolic fraction, accumulated even more strongly in the nucleus (Fig. 3C), suggesting that there may by a transport of cytosolic S1P to the nucleus. In

this view, it was recently reported by Ihlefeld et al [36]. that in S1P lyase knockout fibroblasts, S1P also accumulated in the nucleus although the primary site of S1P accumulation is at the ER. Furthermore, it was shown by Maceyka et al [37]. that SK-2, especially after serum starvation of cells, is localized at internal membranes including the ER, and mediates a pro-apoptotic response. Interestingly, when SK-1 was genetically mutated to target the ER, SK-1-derived S1P also mediated increased apoptosis. Thus, these authors concluded that the site of S1P generation, notably at the ER, is important for a pro-apoptotic effect. Additionally, it was reported that also in cerebellar granule cells, especially S1P generated by SK-2 exerted an apoptotic effect [38]. In our study, we have approached whether the nuclear accumulation of S1P and sphingosine, and the very high level of ceramide, may be due to a major contamination of purified nuclei with ER. Notably, the ER and the nuclear envelop are coupled by a common lipid monolayer being continuous from the nucleus to the ER. We found that the ER marker calreticulin predominantly stained positive in the cytosolic/particulate fraction, with only a minor band (<10%) in the nuclear fraction (Fig. 3A). However, we cannot definitely exclude that certain ER lipids remain at the nucleus during the extraction procedure whereas calreticulin may stay at the main ER body.

The function of SK-2 in proliferation and apoptosis may also be tissue- and cell-specific. In this view, a recent study on dengue virus-induced liver injury showed that SK-2 is a critical mediator of dengue virus-induced apoptosis of hepatic cells. Downregulating SK-2 by siRNA in various hepatic cell lines, reduced dengue virus-induced caspase-9 and the intrinsic pathway of apoptosis without affecting the extrinsic pathway [39]. This study revealed the same apoptotic pathway to be affected by SK-2 as found in our study on renal mesangial cells.

Mesangial cell proliferation and apoptosis are both important events in the course of mesangioproliferative glomerulonephritis. Mesangial cell apoptosis is a very early and acute event in the disease being triggered by factors released by invaded immune cells. It is hypothesized that during this lytic phase, mesangial cells start to release factors, including mitogens like PDGF and ATP [40-42], [18] that in turn promote proliferation. This then propels the disease into the hyperproliferative phase. Thus, it is presently unclear whether therapeutic approaches to reduce apoptosis or rather to reduce proliferation are more favourable to treat the disease. It may be assumed that the first step in the disease, i.e. apoptosis of mesangial cells, is the more important step to prevent the sequelae of the disease. In that case, knowing that loss or inhibition of SK-2 protects from apoptosis, it is tempting to speculate that inhibition of SK-2 may have a beneficial effect in the disease course. Certainly, further studies are needed to unravel the impact of SK-2 and its inhibition *in vivo* for chronic inflammatory and proliferative kidney diseases.

In this view, it was recently reported that SK-2-deficient mice are protected from folic acid-, unilateral ischemia/reperfusion, and unilateral ureter obstruction (UUO)-induced kidney injury and subsequent interstitial fibrosis [19, 43]. In the case of folic acid-induced interstitial fibrosis, the protective effect of SK-2 knockout was mechanistically due to increased interferon-γ production by infiltrating immune cells [43]. Interestingly, these authors also showed that SK-2-deficient splenic T lymphocytes were hyperproliferative and as a consequence produced more interferon-γ [43]. These data are evocative of our previous study showing that SK-2-deficient renal mesangial cells are also hyperproliferative [23] and of the study of Liang and colleagues showing that SK-2-deficient mice develop more severe colitis and colitis-associated colon cancer [28]. In both of these studies, the authors argued that loss of SK-2 leads to a compensatory upregulation of SK-1 with a concomitant production of S1P which may exert a mitogenic effect.

In a complementary approach, hSK2-tg mice upon ureter ligation developed a more severe interstitial fibrosis with enhanced Smad2 and Smad3 activities and multiple fibrotic markers, but downregulated Smad7 [19]. It was speculated that sphingosine could be one of the regulators of Smad7 expression, but more detailed mechanistic studies were not performed.

Notably, increased tubular apoptosis is a typical early feature in the UUO model [44, 45] and it is well possible that parts of the protection seen by loss of SK-2 is due to increased

tubular cell survival and vice versa, that the more severe fibrosis in hSK2-tg is due to accelerated tubular apoptosis.

In summary, our data demonstrated a clear pro-apoptotic and anti-proliferative function of SK-2 in renal mesangial cells which may have impact on pathophysiological processes underlying proliferative kidney diseases.

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Disclosure Statement

The authors declare no conflicts of intersts.

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